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Effect of heat processings on degradation of organophosphorus compounds on preparation of milk products

Sujatha Singh, Nelapati Krishnaiah and Thirtham Madhava Rao

Abstract
Food safety is the major concern worldwide, at public health point of view any contamination of foods will have the deleterious impact on human health. In developing country, like India where the pesticides are used widely to increase the agricultural productivity which in turn, the residues are left over in variable extents in different foods depending upon the nature of pesticide, type of molecule and portion of food material and also in different environmental matter. The present study was undertaken to study the effect of heat processings on the residue levels of various Organophosphorus compounds (Dichlorvos, Diazinon, Dimethoate, Chlorpyrifos, Malathion, Methyl Parathion) in both natural and spiked samples of milk on preparation of various milk products such as fermented products (curd and yoghurt),in desiccated product (khoa) and other coagulated product like paneer. Both natural and spiked samples of milk were processed by adopting the quick, easy, cheap, effective, rugged and safe (QuEChERS) method were analysed on Gas Chromatography – Electron Capture Detector. Among the various products, fermented products proved to be highly effective on degradation of residue levels of organophosphorus compounds than khoa and paneer.

Keywords: Degradation, organophosphorus compounds, Chlorpyrifos, Methyl Parathion

1. Introduction
Indian Agriculture is the backbone of country's economy and sole source of earnings in rural areas of India. More than 58% of its population is dependent on agriculture. Due to different diverse agro climatic conditions in India, intensive agricultural farming, however was not sufficient to meet the requirements for the ever increasing population, mainly due to production losses during growing, harvesting and storage. Moreover, integrated pest management methodologies like neem based insecticides and bio pesticides are recommended, still the farmers rely more on chemical insecticides because of their easy availability, cheap, immediate and spectacular effect [1]. Food security is one of the major concerns for India, where the land area under cultivation was limited, and most of the gains in agricultural production have come from increased productivity through two major inputs i.e. fertilizers and pesticides.

Due to indiscriminate usage of registered and nonregistered pesticides, the important pests are targeted and non- targeted species are also get effected i.e. 0.1% of applied pesticide is used for pest control while 99.9% of pesticides ends up in the environment by seeping out through soil, water and drifted to long distances affecting not only public health but also creating an ecological imbalance throughout the food chains [2]. The pesticide enter into the environment as 'residue' into the terrestrial and aquatic food chains which will concentrate and exert a long term adverse health affect [3]. "Pesticide residues" means any specified substances in food, agricultural commodities or animal feed resulting from use of pesticides. The term includes any derivatives of pesticides such as conversion products, metabolites, reaction products and impurities considered being of toxicological significance [4]. Usage of pesticides are usually authorized and their toxic pattern is evaluated to determine the acceptable exposure for both farm workers and consumers but excessive use and misuse in developing countries is common practice [5]. Food, mainly fatty foods, meat, fish and dairy product have been identified at the primary immediate intake routes of pesticides in general population [6]. Most of the time, the presence of pesticide residues in animal feeds is the main source of pesticide contamination of dairy products, but other factors also includes environmental contamination, application of pesticides on farm animals for ectoparasite removal and accidental spills [7].

The occurrence of pesticides residues in milk and dairy products is a matter of public health concern, since they are widely consumed by infants, children and adults throughout the world. Organophosphorus Pesticides (OPP) are widely used to control plagues in plantations and also used directly to control parasites in cattle. The inappropriate use and handling of these substances may leads to accumulate as residues in feed supplied to cattle and these, when metabolized, are deposited in milk, fat and muscles. Pesticide residual analysis in milk and in their products are more important to study the exposure levels of residual contamination in common public. The concept of food processing is very much important to study the various metabolites, identify breakdown or reaction products generated by the different food processing methods and to find out the levels of residue in processed products and to evaluate dietary-exposure calculations [8]. Effect of food processing on pesticide residue levels may be influenced by the physical location as well as the physio-chemical properties such as solubility, volatility and thermal degradation of pesticides [9].

2. Materials and Methods

Raw Milk samples were collected from local markets of Hyderabad. Later both natural and spiked milk samples (with respective OPP compounds) were processed by adopting quick, easy, cheap, effective, rugged and safe (QuEChERS) method for analysis of pesticide residues. Method was validated with recovery of 70-120% is the acceptable limit for the analysis of milk samples on Gas Chromatography-Electron Capture Detector.

2.1 Chemicals and Reagents

Acetonitrile (ACN), Acetone, n- Hexane, Anhydrous Sodium Sulphate, Sodium Acetate, Primary Secondary Amines (PSA), C_{18} (octadecylsilane) and Magnesium Sulphate (Anhyd.$MgSO_4$) of High Performance Liquid chromatography residue grade obtained from Qualigens and Merck specialities private limited. Analytical standards with >99% purity were obtained from Pesticide Laboratory associated with Professor Jayashankar Telangana State Agriculture University (PJTSAU) and stored in deep freeze maintained at -40˚C.

Table 1: Gc Operating Conditions

1.G C Column	Zebron-ZB-50, Length -30m, 0.25µm film thickness, internal diameter 0.25mm
Column Oven(^{0}C)	280 ^{0}C
Detector temperature	300 ^{0}C
Injector temperature	260 ^{0}C
Injector status	Font injector type split, split ratio 1:10
Carrier gas flow (ml/min)	12.4 ml/min

2.2 Pesticide Analysis

Multi residue analysis of pesticides and sample extraction and cleanup methods were carried out by using QuEChERS method with slight modification [10] i.e. Official AOAC method 2007.01 [11]. 15g of milk sample weighed with 15ml of ACN containing 1% acetic acid followed by addition of 6g of $MgSO_4$ (Anhyd) and 1.5g of sodium acetate. Shake vigorously for 1 min by hand. Later centrifuge the tubes at 5000 RPM for 1 min. After centrifugation, 1 ml of ACN extract will be transferred to minicentrifuge tubes for dispersive solid phase extraction (d SPE) in which 50mg of PSA, 50 mg of C_{18} and 150mg of anhydrous $MgSO_4$were added and mixed the extracts for 20s and again mixed for 30s using a vortex mixer and centrifuged for 5 min at 3000 RPM. Finally 2 ml of clear extract was collected and evaporated under the gentle stream of nitrogen (15psi) using Turbovac LV set at 52^0 C until near dryness up to 20min.The dried residue content was reconstituted in 1 ml of n-Hexane and further analysed in GC-ECD. In control samples, pure and fortified milk samples with standard mixtures were processed with similar protocol and analysed by using same protocol.

2.2.1 Method Validation:

The required quantity of OPP compounds of International standards were prepared from certified reference materials obtained from stock standards prepared in Pesticide Residue laboratory located at PJTSAU Rajendranagar. The OPP Standards were fortified in the representative samples of milk at the rate of 1 ppm. The efficiency of extraction were evaluated based on the recoveries of residues upto the level above 70 to 120% is considered, as acceptable limit. The elution pattern of OPP standards were analysed on the basis of specific retention time for GC-ECD. The limit of detection and limit of quantification of specific OPP compounds was 0.05 ppm respectively. The recovery value were calculated from the calibration curves constructed from the concentration and peak areas of the obtained chromatograms with standards of OPP pesticide. Blank analysis of milk samples was also performed in order to check the different matrix interferences. The residues of pesticides recovered from natural and fortified samples were calculated using the following formula.

$$\text{Residues in ppm} = \frac{\text{Sample peak area}}{\text{Standard Peak area}} \times \text{conc. of std (ppm)} \times \frac{\mu\text{l std. injected}}{\mu\text{l of sample injected}} \times \frac{\text{Final volume of the sample (2 ml)}}{\text{Wt of the sample}}$$

2.3 Statistical Analysis

Data were subjected to one way analysis of variance (ANOVA) to determine the percentage of degradation of organophosphorus compounds in natural and fortified samples of milk.

3. Results and Discussion

The present study was undertaken to estimate the levels of various OPP Compounds (Dichlorvos, Diazinon, Dimethoate, Chlorpyrifos, Malathion, Methyl Parathion) in milk on preparation of various milk products like curd, Yoghurt, khoa, and paneer and the effect of heat processing methods on the residue levels were analysed by testing both natural as well as spiked samples. Limit of detection (LOD) and limit of quantification (LOQ) were estimated to determine the ability of the analytical method to detect and quantify the lowest concentration of the analyte in the given sample. The minimum limit of detectability and minimum limit of

quantification obtained in this study was 0.05 ppm for OPP. The LOD and LOQ in the present study were well below their respective MRLs indicating that, this method was able to detect the given pesticide at a sufficiently low level. Almost similar or low levels were reported for OPP in milk and meat samples [12].

The levels of Dichlorvos, Dimethoate, Diazinon, Chlorpyrifos and methyl-parathion compounds were recorded at below detectable level in the samples while malathion concentration levels were high i.e. 0.1398 ppm. The residue levels of Organophosphorus compounds in after heat treatments were presented in table no.2.

3.1 Degradation of Organophophorus on conversion of milk into curd and Yoghurt

3.1.1 Residue Levels of Dichlorvos

The residue levels of Organophosphorus compounds in curd and Yoghurt after 24 and 48 hours of incubation were presented in table no.2 & 3.

The Dichlorvos content in raw milk was BDL, in spiked milk samples (1ppm), it was 0.870 ppm, reduced to 0.0575 ppm accounting 93.39% of degradation in curd after 24 hours and BDL in 48 hrs of incubation. In spiked milk sample (1ppm), the Dichlorvos content was 0.870 ppm reduced to BDL after 24 hours of incubation in Yoghurt. The Dichlorvos content in spiked milk and curd after 24 hours incubation differed significantly ($p<0.01$).Heat treatment reduces the Dichlorvos content by 84 - 90% in preparation of Tofu [13]. Dichlorvos is an unstable pesticide and very volatile, it may get vapourized by heat during cooking stages [14].

3.1.2 Residue levels of Dimethoate

The Dimethoate content in milk was BDL, in spiked milk samples (1ppm), it was 0.840ppm, reduced to 0.116 ppm in curd, accounting 86.19% of degradation in 24 hours of incubationwhile in 48 hour of incubation the residue levels of Dimethoate content was BDL. The residue levels differ significantly between spiked milk samples and in curd incubated for 24 hours of incubation. In spiked milk samples (1ppm), Dimethoate content was 0.840 ppm reduced to BDL in yoghurt after 24 hours and 48 hours of incubation. 86.50% of degradation of Dimethoate, which was almost similar to the present study and also revealed that fermentation was the best method compared to other processing methods on degradation of Dimethoate content in foods [15]. The organophosphorus pesticides in foods were degraded affectively by fermentation [16, 17].

Table 2: Effect of fermentation on Organophosphorus Compounds in milk

Name of the Pesticide		Curd				
		Raw Milk (ppm)	24 hrs	Deg (%)	48 hrs	Deg (%)
Dichlorvos	Natural	BDL	BDL		BDL	
	Spiked	0.870 ± 0.49^{a}	0.0575± 0.007^{b}	93.39	BDL	-
Dimethoate	Natural	BDL	-	-	-	-
	Spiked	0.840 ±0.45^{a}	0.116 ± 0.007^{b}	86.19	BDL	-
Diazinon	Natural	BDL			-	-
	Spiked	0.8723 ±0.48^{a}	0.1419 ± 0.004^{b}	83.72	0.055±0.003^{c}	93.69
Chlorpyrifos	Natural	BDL	-	-		
	Spiked	0.884 ±0.44^{a}	0.2321± 0.004^{b}	73.76	0.1427±0.024^{c}	83.86
Malathion	Natural	0.1398 ±0.014^{a}	0.0540 ±0.031^{b}	61.37	BDL	-
	Spiked	0.877 ±0.021^{a}	0.0764 ±0.016^{b}	91.28	0.0520 ±0.01^{b}	94.07
methyl Parathion	Natural	BDL				
	Spiked	0.890 ±0.022^{a}	0.2750 ±0.024^{b}	69.12	0.1250±0.021^{c}	85.96

Residual levels bearing different superscripts a, b, c, d and e horizontally differed significantly ($p<0.01$)

Table 3: Effect of fermentation on Organophosphorus Compounds in milk

			Yoghurt			
Pesticide Name		Raw Milk (ppm)	24 Hrs	Deg (%)	48 Hrs	Deg (%)
Dichlorvos	Natural	BDL	BDL	-	BDL	-
	Spiked	0.870 ±0.49	BDL	-	BDL	-
Dimethoate	Natural	BDL	BDL	-	BDL	-
	Spiked	0.840 ±0.45^{A}	BDL	-	BDL	-
Diazinon	Natural	BDL	-	--		-
	Spiked	0.8723 ±0.48^{A}	0.085 ±0.005^{B}	90.25	BDL	
Chlorpyrifos	Natural	BDL	-	-	-	-
	Spiked	0.884 ± 0.44^{A}	0.2114 ±0.023^{B}	76.10	0.1520 ±0.025^{B}	82.81
Malathion	Natural	0.1398 ±0.014^{A}	0.0510 ±0.001^{B}	63.51	BDL	
	Spiked	0.877 ±0.46^{A}	0.0545 ±0.012^{B}	93.37	BDL	-
Methyl Parathion	Natural	BDL	-	-	-	-
	Spiked	0.8908 ±0.45^{A}	0.227 ±0.012^{B}	74.42	0.1459 ±0.012^{C}	83.62

Residual levels bearing different superscripts A and B horizontally differed significantly ($p<0.01$)

3.1.3 Residual levels of Diazinon

The Diazinon content in milk was BDL, in spiked milk samples (1ppm), the Diazinon content was 0.8723 ppm, reduced to 0.1419 and 0.055 ppm, accounting 83.72 and 93.69% of degradation in 24 and 48 hours of incubation respectively. The Diazinon content in raw milk and in treated milk significantly ($p<0.01$) differed. The content of Diazinon content in spiked milk and its curd after 24 and 48 hours of incubation differed ($p<0.01$) significantly. In spiked milk samples (1ppm), Diazinon content was 0.8723 ppm reduced to 0.085 ppm in yoghurt, accounting 90.25% of degradation in 24 hours and it was BDL after 48 hour of incubation. The

content of Diazinon content in spiked milk and in yoghurt after 24 of incubation differed ($p<0.01$) significantly. Lactic Acid Bacteria as starters or prolonged periods of incubation could accelerate the degradation of the Organophosphorus compounds [18].

3.1.4 Residual levels of Chlorpyrifos

The Chlorpyrifos content in milk was BDL, in spiked milk samples (1ppm), Chlorpyrifos content was 0.884 ppm, reduced to 0.2321 and 0.1427ppm, accounting 73.76 and 83.86% of degradation respectively. The content of Chlorpyrifos in spiked milk and its curd after 24 and 48 hours of incubation differed ($p<0.01$) significantly. In spiked milk samples (1ppm), Chlorpyrifos content(0.8846) ppm, reduced to 0.2114 ppm in yoghurt, accounting 76.10% of degradation in 24 hours of incubation while in 48 hour of incubation the residue levels of Chlorpyrifos content was 0.1520 ppm with 82.81% of degradation. The content of Chlorpyrifos in spiked milk differed ($p<0.01$) significantly with Yoghurt after 24 and 48 hours of incubation. The content of Chlorpyrifos in Yoghurt prepared from spiked milk incubated at 24 and 48 hours of incubation were nonsignificant.

3.1.5 Residual levels of Malathion

The Malathion content in raw milk was 0.1398 ppm, reduced to 0.0540 ppm after 24 hours of incubation in curd, accounting for 61.37% degradation and further reduced, to BDL after 48 hours. In spiked milk samples (1ppm), Malathion content was 0.877 ppm, reduced to 0.0764 and 0.0520ppm, accounting for 91.28 and 94.07% of degradation in 24 and 48 hours of incubation. The content of Malathion in raw milk and in curd after 24 hrs of incubation differed ($p<0.01$) significantly in both spiked and natural samples of milk, but not significant between 24 and 48 hours of incubation. The Malathion content in raw milk was 0.1398 ppm, reduced to 0.0510 ppm in Yoghurt after 24 hours of incubation, accounting 63.51% degradation, whereas the content was BDL after 48 hours of incubation. In spiked milk samples (1 ppm), Malathion content was 0.8771 ppm, reduced to 0.0545 ppm, accounting for 93.37% of degradation in 24 hours of incubation while after 48 hour of incubation it was BDL. The content of Malathion in both spiked and natural samples of milks differed ($p<0.01$) significantly with yoghurt after 24 hours of incubation. 97.17% of degradation in spiked buffalo milk samples was observed similar to the present study findings in curd [15]. High heat treatments and prolonged fermentation degrade pesticides faster in the order of methyl parathion > denthion > malathion > trichlorfon> dimethoate > monocrotophos > phorate [18]. The behavior of residues in fermentation can be rationalized in terms of the physical-chemical properties of the pesticide and the nature of the process [19].

3.1.6 Residual levels of Methyl Parathion

The Methyl Parathion content in milk was BDL. In spiked milk samples (1ppm), it was 0.890 ppm, reduced to 0.2750 and 0.1250 ppm, accounting 69.12and 85.96% of degradation after 24 and 48 hours of incubation. The Methyl Parathion content in the spiked milk and in curd after 24 and 48 hour of incubation were differ significantly ($p<0.01$). In spiked milk samples (1ppm), Methyl Parathion content was 0.890 ppm, reduced to 0.227 ppm in yoghurt accounting 74.42% of degradation in 24 hours of incubation, while in 48 hour of incubation it was 0.1459 ppm with 83.62% of degradation. Methyl parathion content in spiked milk differed ($p<0.01$) significantly with Yoghurt after 24 and 48 hours of incubation. High heat treatments and prolonged incubation during fermentations are highly effective on pesticide residual reduction in foods [18].

3.2 Degradation of Organophophorus on conversion of milk into khoa and paneer

The residue level of different OPP compounds in khoa and paneer presented in table no.4.

Table 4: Residual levels of Organophosphorus compounds on conversion of milk into products

Name of The Pesticide		Raw Milk (ppm)	Khoa (mg/Kg Fat Basis)	Deg	Paneer (mg/Kg Fat Basis)	Deg (%)
Dichlorvos	Natural	BDL	-	-	-	-
	Spiked	19.02 ±0.55^{aA}	BDL	-	10.9 ± 0.002^{B}	42.69
Dimethoate	Natural	BDL	-	-	-	-
	Spiked	19.02 ±0.55^{aA}	8.11 ± 0.004 ^{b}	57.36	7.71 ± 0.005^{B}	59.46
Diazinon	Natural	BDL	-	-	-	-
	Spiked	19.26 ±0.57^{aA}	8.33 ± 0.021^{b}	56.74	7.77 ± 0.005^{B}	59.65
Chlorpyrifos	Natural	BDL	-	-	-	-
	Spiked	17.38 ±0.23^{aA}	10.36 ± 0.008^{b}	40.39	8.17± 0.003^{B}	52.99
Malathion	Natural	3.106 ±0.014^{aA}	BDL	-	BDL	-
	Spiked	19.23 ±0.65^{aA}	10.97 ±0.004^{b}	42.95	10.12 ±0.005^{B}	47.37
methyl Parathion	Natural	BDL	-	-	-	-
	Spiked	19.49 ±0.35^{aA}	10.11 ±0.018^{b}	48.12	7.035 ± 0.0061^{B}	63.90

Residual levels bearing different superscripts a, b, c, d and e horizontally differed significantly ($p<0.01$)
Residual levels bearing different superscripts A and B horizontally differed significantly ($p<0.01$)

3.2.1 Residual levels of Dichlorvos

The content of Dichlorvos in raw milk sample was BDL, whereas in spiked milk testing 19.02mg/Kg fat basis was reduced to BDL in khoa, while it was reduced to 10.90 mg/kg fat basis in paneer accounting 42.69% of degradation. 50.96% of degradation in coagulated product [15] which was almost similar to the present study, which was attributed to binding small amount of pesticide to the rennet enzyme [20] or to the chemical nature of the pesticide [21].

3.2.2 Residual levels in Dimethoate

The content Dimethoate in milk sample was BDL, whereas spiked milk testing 19.02 mg/Kg fat basis was reduced to 8.11 mg/ kg fat basis accounting for 57.36% of degradation in khoa, while it was reduced to 7.71 mg/kg fat basis in paneer, accounting for 59.46% of degradation. The residue level in spiked milk differed significantly ($p<0.01$) with residual levels in both khoa and paneer.

3.2.3 Residual levels in Diazinon
The Diazinon content was BDL, where as in spiked milk testing 19.26 mg/Kg fat basis, was reduced to 8.33 mg/kg fat basis and 7.77mg/kg fat basis, accounting 56.74 and 59.65% of degradation in khoa and paneer respectively.

3.2.4 Residual levels of Chlorpyrifos
The content Chlorpyrifos in milk sample was BDL, whereas spiked milk testing 17.38 mg/Kg fat basis, reduced to 10.36 and 8.17 mg/ kg fat basis, accounting for 40.39% and 52.99% of degradation in khoa and paneer respectively.

3.2.5 Residual level of Malathion
The Malathion residue in raw milk was 3.106 mg/kg fat basis, reduced to BDL in both khoa and paneer, whereas spiked milk testing 19.23mg/kg fat basis, reduced to 10.97 and 10.12 mg/ kg fat basis accounting for 42.95% and 47.37% of degradation in khoa and paneer respectively. 85.02% of degradation in paneer [15] which was higher than the present study. Coagulation process causes degradation of malathion [18] as compared with the other treatments, which may be attributed to binding of small amount of pesticide to the rennet enzyme [19] or to the physio chemical nature of the pesticide [8, 19, 21].

3.2.6 Residual level of Methyl parathion
The Methyl parathion residue in raw milk was at BDL, but in spiked milk testing 19.49 mg/Kg fat basis was reduced to 10.11 and 7.035 mg/ kg fat basis, accounting for 48.12 and 63.90% of degradation in khoa and paneer respectively.The Khoa and Paneer prepared from spiked milk differed significantly ($p<0.01$) in terms of Dichlorvos, Dimethoate, Diazinon, Chlorpyrifos, Malathion and Methyl Parathion residues. The OPP pesticides in foods were affected by fermentation, heat treatments and drying [16, 17]. Food processing leads to large reduction in organophosphorus pesticide levels [8, 19]. Pasteurization, sterilization and fermentation will reduce the pesticide residues effectively in foods on processing [22].

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